American Journal of Medical Genetics 64:399–403 (1996)

Lack of Expansion of Triplet Repeats in the FMR1, FRAXE, and FRAXF Loci in Male Multiplex Families With Autism and Pervasive Developmental Disorders

Jeanette J.A. Holden, Maggie Wing, Maryse Chalifoux, Colin Julien-Inalsingh, Chris Schutz, Paula Robinson, Peter Szatmari, and Bradley N. White

Department of Psychiatry, Queen's University, Kingston (J.J.A.H., C.J-I.); Cytogenetics and DNA Research Laboratory, Ongwanada Resource Centre, Kingston (J.J.A.H., M.W., M.C.); Departments of Biology (C.S., P.R., B.N.W.) and Psychiatry (P.S.), McMaster University, Hamilton, Ontario, Canada

Sib, twin, and family studies have shown that a genetic cause exists in many cases of autism, with a portion of cases associated with a fragile X chromosome. Three folate-sensitive fragile sites in the Xq27→Xq28 region have been cloned and found to have polymorphic trinucleotide repeats at the respective sites; these repeats are amplified and methylated in individuals who are positive for the different fragile sites. We have tested affected boys and their mothers from 19 families with two autistic/PDD boys for amplification and/or instability of the triplet repeats at these loci and concordance of inheritance of alleles by affected brothers. In all cases, the triplet repeat numbers were within the normal range, with no individuals having expanded or premutation-size alleles. For each locus, there was no evidence for an increased frequency of concordance, indicating that mutations within these genes are unlikely to be responsible for the autistic/PDD phenotypes in the affected boys. Thus, we think it is important to retest those autistic individuals who were cytogenetically positive for a fragile X chromosome, particularly cases where there is no family history of the fragile X syndrome, using the more accurate DNA-based testing procedures. © 1996 Wiley-Liss, Inc.

KEY WORDS: autism/PDD, Asperger syndrome, fragile X syndrome, FMR1

INTRODUCTION

Autism is a developmental disability characterized by impairments in reciprocal social interaction, and verbal and non-verbal communication, and a pattern of repetitive, stereotypic activities. It represents the most extreme form of a spectrum of conditions termed the "pervasive developmental disorders" (PDD) that share these manifestations but differ in natural history, number of symptoms, or pattern of behaviours [Szatmari, 1992]. The usual onset of autism is before the age of 3 years and occurs in 4–10/10,000 children, with a sex ratio of about 4:1 affected males:females [Bryson et al., 1988].

Recent major reviews of sib, twin, and family studies recognize a genetic cause in many cases of autism [Silliman et al., 1989; Rutter et al., 1990]. The findings include an increased risk to sibs of autistic probands (mean is 3%) and differences in concordance rates among monozygotic (64%) and dizygotic (9%) twins. About 10% of cases of autism [Rutter et al., 1990] co-occur with other well-described disorders, such as tuberous sclerosis, neurofibromatosis, and phenylketonuria or with certain viral diseases, such as congenital rubella and cytomegalovirus. Whether the remaining 90% of cases (so-called "idiopathic" cases) are genetically homogeneous or heterogeneous remains to be determined.

Perhaps one of the most intriguing genetic associations made during the early 1980's to early 1990's was the observation, albeit controversial, of an apparent co-occurrence of autism and the fragile X syndrome [for example, Brown et al., 1982, 1986; Meryash et al., 1982; Watson et al., 1984; Blomquist et al., 1985; Einfeld et al., 1989; Cohen et al., 1991; Piven et al., 1991]. Some of the behavioral characteristics of males with the fragile X syndrome are described as "autistic"or "autistic-like." Further, chromosome studies on autistic individuals revealed that varying proportions of subjects tested positive for chromosome fragility in Xq27→Xq28.

The Xq27→Xq28 region harbours three rare folate-sensitive fragile sites. FRAXA is the site of the FMR1 gene, which is mutated in individuals who have the

Received for publication October 2, 1995; revision received December 28, 1995.

Address reprint requests to Dr. Jeanette J.A. Holden, Cytogenetics and DNA Research Laboratory, Ongwanada Resource Centre, 191 Portsmouth Avenue, Kingston, Ontario K7M 8A6, Canada.

fragile X syndrome. Most mutations that result in the fragile X syndrome arise through amplification or expansion of a hypervariable CGG-trinucleotide repeat in the 5′ untranslated region of the FMR1 gene [Verkerk et al., 1991; Yu et al., 1991; Oberlé et al., 1991]. Amplification coupled with methylation of a CpG island and the CGG-repeats in affected males, results in fragility at the FRAXA site. The identification of similar repeats and their methylation, when extensively expanded, have been shown to result in fragility at the nearby FRAXE and FRAXF fragile sites [Knight et al., 1993; Parrish et al., 1994; Ritchie et al., 1994].

For all three folate-sensitive sites in Xq27→q28, DNA tests for amplification can now been done to determine precisely whether there is triplet repeat expansion at one of these cytogenetically difficult to distinguish fragile sites. We have combined these DNA-based tests with cytogenetic analyses to examine 19 families, each with two autism/PDD brothers, and report our findings here.

MATERIALS AND METHODS

Subjects

The multiplex families were recruited in three ways. First, families with more than one child with PDD were identified among those attending the PDD clinic at Chedoke-McMaster Hospitals. Second, a questionnaire was sent to parents who were members of the Autism Society of Ontario, inquiring whether there was a second child in the family affected with autism, PDD, or developmental disability. Third, a letter was sent to all children's mental health and social service agencies in southern Ontario that serve children with developmental disabilities, with clinicians being asked to complete a brief questionnaire on any autistic/PDD child under their management, indicating whether that child had a sib with autism, PDD, or developmental disability.

Parents in potential multiplex families were asked whether their children displayed any PDD-like behaviours in their development. The children were assessed using the Autism Diagnostic Interview (ADI) [Le Couteur et al., 1989] and the Autism Diagnostic Observation Schedule (ADOS) [Lord et al., 1989]. In addition, the Autism Behavior Checklist (ABC) [Krug et al., 1980] was completed on each child and clinical records containing information pertaining to diagnosis and clinical assessments were examined. All information was reviewed to make a diagnosis of autism (severe impairments in qualitative social interaction with parents and peers; deficits in verbal and non-verbal communication, including deviant language development; and a pattern of restrictive, repetitive behaviours), Asperger syndrome (no clinically significant cognitive or language delay, but qualitative impairments in reciprocal social interaction and repetitive stereotypic behaviours), or atypical PDD (fewer behaviours in total than needed to receive a diagnosis of autism), according to ICD-10 [WHO, 1992] and DSM-IV [American Psychiatric Association, 1994] criteria. Of 33 potential multiplex families, 11 elected not to participate; DNA samples and clinical assessments were available on members of 19 of the remaining families. Psychometric testing, using the Leiter Performance Scales [Levine, 1986], is in progress and results were available for some of the affected children.

Chromosome Testing

Cytogenetic analyses were performed on lymphocytes from affected males only, cultured in modified RPMI 1640 (for fragile X studies) and alpha-MEM with FUdR [Glover, 1981] to determine whether there were any chromosome abnormalities and whether fragility was expressed at the FRAXA, FRAXE, or FRAXF sites. Fifty to one hundred cells were examined per individual; whenever possible, 50 cells were scored for each culture condition. Cytogenetic studies were possible on 25 of the 38 affected boys.

FMR1, FRAXE, and FRAXF Trinucleotide Repeat Testing

Blood was drawn from all affected children and both parents and DNA was extracted using conventional methods, or PCR amplification was done directly from dried blood spots as described in Holden et al. [1996]. All samples were tested for amplification of the trinucleotide repeats in the respective loci according to previously described PCR protocols [FMR1: Fu et al., 1991; FRAXE: Knight et al., 1993; FRAXF: Parrish et al., 1994; Ritchie et al., 1994]. Triplet repeat numbers for the FRAXF repeat were as calculated by Ritchie et al., who found three or four copies of a GCCGTC array adjacent to the GCC-repeat region described by Parrish et al. [1994]. Thus, for example, repeat numbers of 14 could represent either of the following configurations: $(GCCGTC)_3(GCC)_8$ or $(GCCGTC)_4(GCC)_6$. Since Parrish et al. [1994] did not observe variation in the number of GCCGTC arrays among those they sequenced, they concluded that all variation resulted from the number of GCC-repeats. For comparisons of our results with those of Parrish et al., it is necessary to add six to their triplet repeat numbers. Southern blotting of EcoR1/EagI digests and probing with pE5.1 was performed for all cases of FMR1 CGG-repeat numbers $\geq$35 repeats and of HindIII digests and probing with OxE20 for all cases of FRAXE GCC-repeat numbers $\geq$22 repeats to test for instability of the alleles.

RESULTS

Nineteen families, each with two boys with autism or a related PDD, were studied, with diagnoses including autism (28/38), Asperger syndrome (7/38), atypical PDD (3/38) (Fig. 1). Seven of 25 boys tested had an IQ $<$70. None of the boys had a clinical phenotype suggestive of the fragile X (FRAXA) syndrome.

Chromosomal fragile site studies were performed on 25 males from 14 families. A single cell in one subject had a FRAXA fragile site. Two subjects each showed fragility in 2/100 cells at either FRAXE or FRAXF, and one subject had a single cell with possible fragility at FRAXE/F. Three subjects each had one cell with a fragile site at FRAXD.

Figure 1 summarizes the CGG- and GCC-repeat numbers for the FMR1, FRAXE, and FRAXF loci, respectively, for all mothers and sons. Southern blot analyses

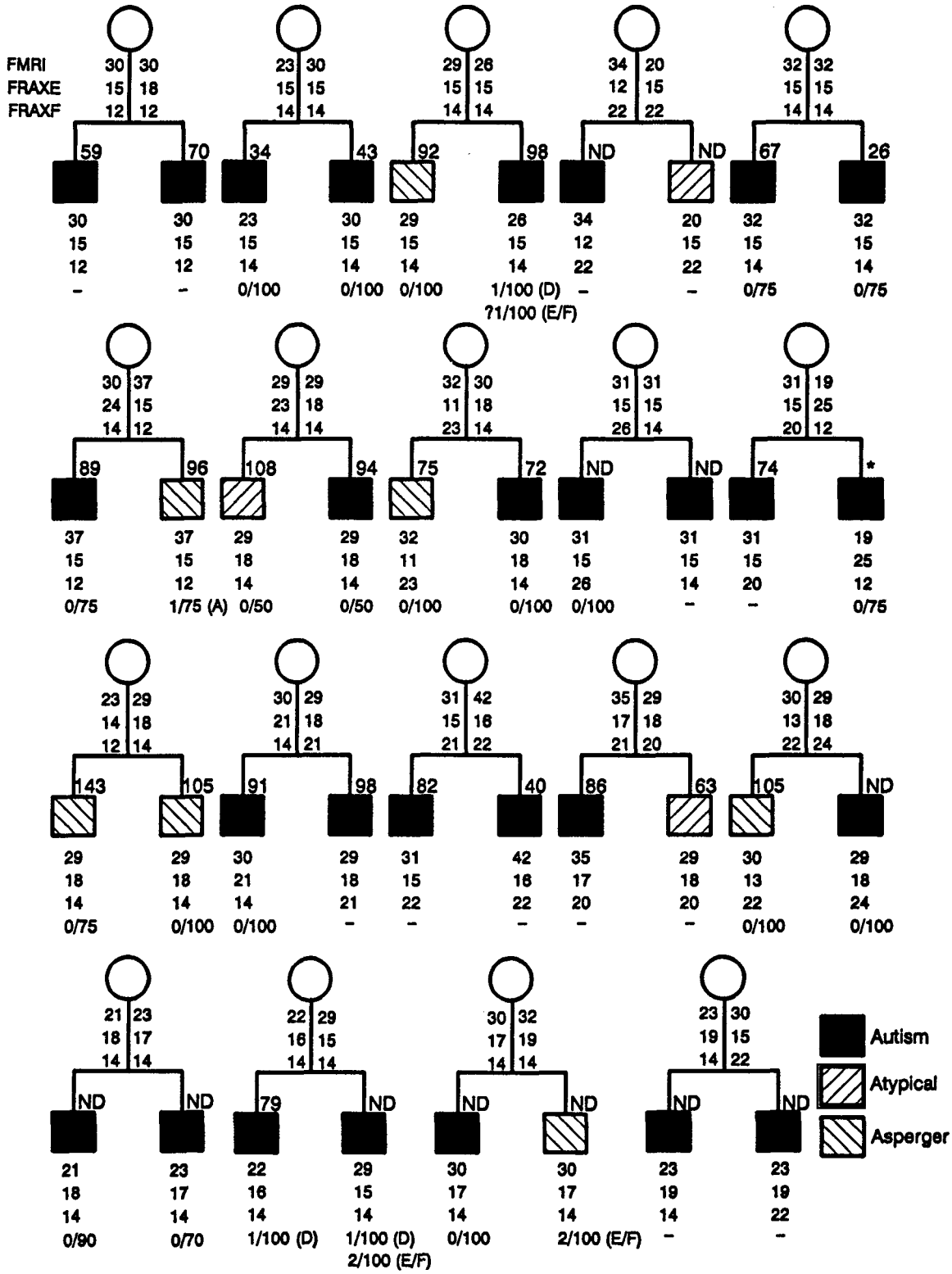

Fig. 1. FMR1, FRAXE, and FRAXF triplet repeat numbers and cytogenetic fragile sites seen in autistic/PDD brothers from 19 families. The numbers indicate the number of triplet repeats at the FMR1, FRAXE, and FRAXF sites. For the affected boys, the number of cells with a fragile site at Xq27.2 (D: FRAXD), Xq27.3 (A: FRAXA), or at Xq28 (E/F: FRAXE or FRAXF) are indicated out of the total number of cells examined for that individual. IQs are indicated at the upper right of pedigree symbols (ND, not yet determined; *, unable to be tested).

TABLE I. Tests for Concordance at FMR1, FRAXE, and FRAXF in Affected Sib Pairs

	FMR1 concordance	χ^2 (ns)	FRAXE concordance	χ^2 (ns)	FRAXF concordance	X^2 (ns)
All	4/15	3.27	6/15	0.60	4/10	0.40
AUT	1/7	3.57	2/7	1.29	1/5	1.8
AUT + Atyp	1/9	5.44	3/10	1.60	2/6	0.67
AUT + Asp	4/13	1.92	5/12	0.33	3/9	1.0

showed normal male patterns for all autistic/PDD males and normal female patterns in all mothers with FMR1 CGG-repeats ≥35 and FRAXE GCC-repeats ≥22 repeats using both single (EcoR1) and double (EcoR1/EagI) digests for FMR1 and HindIII digests for FRAXE. Two points are evident in Figure 1: 1) All repeat sizes are within the normal ranges for each locus; and 2) the proportions of concordant and discordant sibs when the mothers were informative are 4:11 for FMR1, 6:9 for FRAXE and 4:6 for FRAXF, when all diagnoses were considered (Table I). When one considers each diagnostic subtype separately or in different combinations, there is also no indication of increased concordance.

DISCUSSION

Both the cytogenetic and molecular studies on the FMR1, FRAXE, and FRAXF loci in 19 families, each with two autistic/PDD sons, indicate that mutations within the triplet repeats located at these sites are not responsible for the clinical phenotypes of autism, Asperger syndrome or atypical autism in the multiplex families studied. In two cases, low percentages of fragile sites were seen at FRAXE/FRAXF, which are cytogenetically indistinguishable except by using fluorescence in situ hybridization with a probe located between these two sites [Hirst et al., 1993]. The significance of the low percentages of FRAXE/F fragility is unknown, but it may be that there are other fragile sites in this region which have not yet been identified because of their proximity to FRAXA, FRAXE, and FRAXF. FRAXD is a common fragile site, with no known clinical significance. It is also relatively easily distinguished from the folate-sensitive fragile sites in the Xq27→Xq28 region, since it is more proximally located.

The molecular findings for FMR1 are similar to those in a study by Hallmayer et al. [1994], who found no evidence for either amplifications of the FMR1 CGG-repeat or for linkage of the CGG-repeat region or microsatellite loci flanking the CGG-repeat (DXS548, FRAXAC1, and FRAXAC2) to autism/PDD in 35 multiplex families having a total of 79 affected children, including both boys and girls. Our findings of no triplet repeat expansion at FMR1, FRAXE, or FRAXF and the lack of concordance of alleles at these loci in affected sibs, combined with the findings of Hallmayer et al. [1994], strongly suggest that individuals who are ascertained as being autistic have a low probability of having triplet repeat expansion at one of these sites or a mutation linked to this region of the X-chromosome.

The question, then, is how do we resolve the earlier observation that a varying, but significant, proportion of autistic individuals have a "fragile X" chromosome? In cases where the frequency of fragile site expression was low, as seen in our sample, it could be that these represent background fragility or other fragile sites at these same cytogenetic locations. The ability, at this time, to distinguish three fragile sites on the basis of a simple molecular test, provides an opportunity to test whether the X-chromosome fragility seen in previous studies of autistic patients is at FRAXA, FRAXE, or FRAXF. Although there is currently no evidence that FRAXA, FRAXE, or FRAXF triplet repeat expansions or mutations (as seen by the lack of significant concordance between affected brothers) are responsible for autism in multiplex families, it will be important to test isolated cases of autism/PDD for such expansions, since the cause in multiplex families may be different from that in simplex families. In addition, a comparison of the results of FMR1, FRAXE, and FRAXF testing in autistic/PDD individuals with and without developmental disability in the index-cases and/or relatives may clarify the association of autism and fragile sites on the long arm of the X-chromosome.

ACKNOWLEDGMENTS

We thank the following for their contributions in the clinical assessments of the children in this study: Dr. W. Mahoney, Dr. G. Bartolucci, and Dr. S. Bryson. This work was supported by grants from the Ontario Mental Health Foundation (to J.J.A.H. and B.N.W.) and the Medical Research Council of Canada (to P.S., B.N.W., and J.J.A.H.).

REFERENCES

American Psychiatric Association (1994): Diagnostic and statistical manual of mental disorders (DSM-IV), American Psychiatric Association Press, Washington, D.C.

Blomquist HK, Bohman M, Edvinson SO, Gillberg C, Gustavson KH, Holmgren G, Wahlstrom J (1985): Frequency of the fragile X syndrome in infantile autism. Clin Genet 27:113–117.

Brown WT, Jenkins EC, Friedman E, Brooks J, Wisniewski K, Raguthu S, French JH (1982): Autism is associated with the fragile X syndrome. J Autism Dev Dis 12:303–307.

Brown WT, Jenkins EC, Cohen IL, Fisch GS, Wolf-Schein EG, Gross A, Waterhouse L, Fein D, Mason-Brothers A, Ritvo E, Ruttenberg BA, Bentley W, Castells S (1986): Fragile X and autism: A multicenter study. Am J Med Genet 23:341–352.

Bryson SE, Clark BS, Smith I (1988): First report of a Canadian epidemiological study of autistic syndromes. J Child Psychol Psychiatry 29:433–446.

Cohen IL, Sudhalter V, Pfadt A, Jenkins EC, Brown WT, Vietze PM (1991): Why are autism and the fragile X syndrome associated? Conceptual and methodological issues. Am J Hum Genet 48:195–202.

Einfeld S, Molony H, Hall W (1989): Autism is not associated with the fragile X syndrome. Am J Med Genet 34:187–193.

Fu YH, Kuhl DPA, Pizzuti A, Pieretti M, Sutcliffe JS, Richards S, Verkerk AJMH, Holden JJA, Fenwick RG, Warren ST, Oostra BA, Nelson DL, Caskey CT (1991): Variation of the CGG repeat at the fragile X site results in genetic instability: Resolution of the Sherman paradox. Cell 67:1047–1058.

Glover TW (1981): FUdR induction of the X chromosome fragile site: evidence for the mechanism of folic acid and thymidine inhibition. Am J Hum Genet 33:234–242.

Hallmayer J, Pintado E, Lotspeich L, Spiker D, McMahon W, Petersen PB, Nicholas P, Pingree C, Kraemer HC, Wong DL, Ritvo E, Lin A, Hebert J, Cavalli-Sforza LL, Ciaranello RD (1994): Molecular analysis and test of linkage between the FMR-1 gene and infantile autism in multiplex families. Am J Hum Genet 55:951–959.

Hirst MC, Barnicoat A, Flynn G, Wang Q, Daker M, Buckle VJ, Davies KE, Bobrow M (1993): The identification of a third fragile site, FRAXF, in Xq27-q28 distal to both FRAXA and FRAXE. Hum Mol Genet 2:197–200.

Holden JJA, Chalifoux M, Wing M, Julien-Inalsingh C, White BN (1996) A rapid, reliable and inexpensive method for detection of di- and trinucleotide repeat marker and disease loci from dried blood spots. Am J Med Genet 64:313–318.

Knight SJL, Flannery AV, Hirst MC, Campbell L, Christodoulou Z, Phelps SR, Pointon J, Middleton-Price HR, Barnicot A, Pembrey ME, Holland J, Oostra BA, Bobrow M, Davies K (1993): Trinucleotide repeat amplifications and hypermethylation of a CpG island in FRAXE mental retardation. Cell 74:127–134.

Krug DA, Arick J, Almond P (1980): Behavior checklist for identifying severely handicapped individuals with high levels of autistic behavior. J Child Psychol Psychiatry 21:221–229.

Le Couteur A, Rutter M, Lord C, Rios P, Robertson S, Holdgrafer M, McLellan J (1989): Autism diagnostic interview: A standardized investigator based instrument. J Autism Dev Dis 19:363–388.

Levine M (1986): "The Leiter International Performance Scale: A Handbook." Western Psychological Services, U.S.A.

Lord C, Rutter M, Goode S, Heemsbergen J, Jorda H, Mawhood L, Schoplen E (1989): The autism diagnostic observation schedule: A standardized observation of communication and social behavior. J Autism Dev Dis 19:185–212.

Meryash DL, Szymanski LS, Gerald PS (1982): Infantile autism associated with the fragile-X syndrome. J Autism Dev Dis 12:295–301.

Oberle I, Rousseau F, Heitz D, Kretz C, Devys D, Hanauer A, Boue J, Bertheas MS, Mandel JL (1991): Instability of a 550-base pair DNA segment and abnormal methalyzation in fragile X syndrome. Science 252:1097–1102.

Parrish JE, Oostra BA, Verkerk AJMH, Richards CS, Reynolds J, Spikes AS, Shaffer LG, Nelson DL (1994): Isolation of a GCC repeat showing expansion in FRAXF, a fragile site distal to FRAXA and FRAXE. Nat Genet 8:229–239.

Piven J, Gayle J, Landa R, Wzorek M, Folstein S (1991): The prevalence of fragile X in a sample of autistic individuals diagnosed using a standardized interview. J Am Acad Child Adolescent Psychiatry 30:825–830.

Ritchie RJ, Knight SJL, Hirst MC, Grewal PK, Bobrow M, Cross GS, Davies KE (1994): The cloning of FRAXF: trinucleotide repeat expansion and methylation at a third fragile site in distal Xqter. Hum Mol Genet 3:2115–2121.

Rutter M, MacDonald H, LeCouteur A, Harrington R, Bolton P, Bailey A (1990): Genetic factors in child psychiatric disorders, II. Empirical findings. J Child Psychol Psychiatry 31:39–83.

Silliman ER, Campbell M, Mitchell RS (1989): Genetic influences in autism and assessment of metalinguistic performance in sibs of autistic children. In Dawson G (ed): "Autism: Nature, Diagnosis, and Treatment." New York: Guilford Press, pp 225–229.

Szatmari P (1992): The validity of autistic spectrum disorders. J Autism Dev Dis 22:583–600.

Verkerk AJMH, Pieretti M, Sutcliffe JS, Fu Y-H, Kuhl DPA, Pizzotti A, Reiner O, Richards S, Victoria MF, Zhang F, Eussen BE, van Ommen GJB, Blonden LAJ, Riggins GJ, Chastain JL, Kunst CB, Galjaard H, Caskey CT, Nelson DL, Oostra BA, Warren ST (1991): Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell 65:905–914.

Watson MS, Leckman JF, Annex B, Breg WR, Boles D, Volkmar FR, Cohen DJ (1984): Fragile X in a survey of 75 autistic males. New Engl J Med 310:1462.

World Health Organization (1992): "The ICD-10 Classification of Mental and Behavioral Disorders; Clinical Description and Diagnostic Guidelines." Geneva: World Health Organization.

Yu S, Pritchard M, Kremer E, Lynch M, Nancarrow J, Baker E, Holman K, Mulley J, Warren ST, Schlessinger D, Sutherland GR, Richards RI (1991): Fragile X genotype characterized by an unstable region of DNA. Science 252:1179–1181.